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Vitamin-B_{12}-independent methionine synthase from a higher plant (*Catharanthus roseus*)

Molecular characterization, regulation, heterologous expression, and enzyme properties

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Methionine synthases catalyze the formation of methionine by the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine. This reaction is the last step in L-methionine biosynthesis, and it also serves to regenerate the methyl group of *S*-adenosylmethionine, a cofactor required for biological methylation reactions. We describe the cloning, expression and characterization of a methionine synthase from the higher plant *Catharanthus roseus*. cDNAs were identified that encoded a protein of 85 kDa sharing 50% identity with the cobalamin-independent methionine synthase from *Escherichia coli* (MetE) and 41% identity with a partial sequence of a yeast homolog of MetE. The *C. roseus* protein was expressed at high levels in *E. coli*. The enzyme accepts the triglutamate form of methyltetrahydrofolate as a methyl donor but not the monoglutamate form, and it does not require *S*-adenosylmethionine or cobalamin for activity. The properties indicate that the enzyme is a cobalamin-independent methionine synthase (EC 2.1.1.14). In contrast to the *E. coli* MetE, the plant protein does not require phosphate or magnesium ions for activity. Immunoblots of plant extracts showed that the protein was localized in the cytosol, and was present in a variety of plant species. A nutritional downshift of the *C. roseus* cell culture revealed a strong, transient transcriptional activation, but no significant increment in the total level of the protein. The availability of the protein and the cDNA now provide tools to investigate the complexities of methionine biosynthesis in plants.

Keywords. *Catharanthus roseus*; heterologous expression; methionine synthase; methyl cycle; vitamin B_{12}.

Methionine synthase enzymes catalyze the methylation of L-homocysteine to form methionine. The enzymes are required in all organisms for the regeneration of the methyl group of *S*-adenosyl-L-methionine (AdoMet), a compound that serves as the methyl donor for a large number of biological methylations (Fig. 1). The conversion of homocysteine to methionine also constitutes the last step in methionine biosynthesis in organisms that are capable of the *de novo* biosynthesis of this amino acid.

There are two types of methionine synthase that use methyltetrahydrofolate as the methyl donor (reviewed in [1]). One type requires cobalamin (vitamin B_{12}) as a cofactor, and the prototype is the *metH* gene product from *Escherichia coli* (EC 2.1.1.13). MetH-type enzymes are present in organisms that either synthesize vitamin B_{12} (some bacteria) or obtain it from intestinal flora or diet (animals and some enteric bacteria like *E. coli*). The enzymes from animals have not yet been cloned, but the activities are well characterized [2] (and references cited therein). The other type of enzyme is cobalamin-independent, and the prototype is the *metE* gene product from *E. coli* (EC 2.1.1.14). MetE-type enzymes are found in organisms that do not synthesize vitamin B_{12} and do not obtain it from the environment (plants, fungi, and some bacteria). A few bacteria, e.g. *E. coli*, contain both types. MetE and MetH from *E. coli* have been cloned [3, 4], sequenced [5, 6], and extensively studied [7–9]. These two enzymes share no sequence similarity [6].

Methionine synthase enzymes from plants are not well characterized, and the available data were obtained with partially purified enzymes (reviewed in [10, 11]). They are cobalamin-independent (MetE-type) enzymes that use methyltetrahydrofolate polyglutamates as methyl donors but, in contrast to MetE, have been reported to show significant activity with the monoglutamate of methyltetrahydrofolate (CH_3-H_4PteGlu$_1$) as methyl donor. This could reflect the presence of several enzymes, consistent with the detection of activities not only in the cytosol, but also in mitochondria [12] and plastids [13]. However, the presence of these enzymes in plastids and mitochondria has been disputed [14].

We now describe the first cloning and expression of a cobalamin-independent methionine synthase from a higher plant, *Catharanthus roseus*. The protein has significant similarities with MetE from *E. coli*, but some properties are distinctly different. Immunoblots with plant extracts indicate that it is located in the cytoplasm, and closely related proteins are present in all plants

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Abbreviations. AdoMet, *S*-adenosyl-L-methionine; (6*S*)-CH_3-H_4PteGlu$_n$, 5-methyltetrahydropteroylglutamate with *n* glutamyl residues; MetE, cobalamin-independent L-methionine synthase; MetH, cobalamin-dependent L-methionine synthase.

Enzymes. Cobalamin-independent L-methionine synthase (EC 2.1.1.14); cobalamin-dependent L-methionine synthase (EC 2.1.1.13).

Note. The novel nucleotide sequence data reported here have been submitted to the GenBank™/EMBL Data Bank and are available under the accession number X83499.

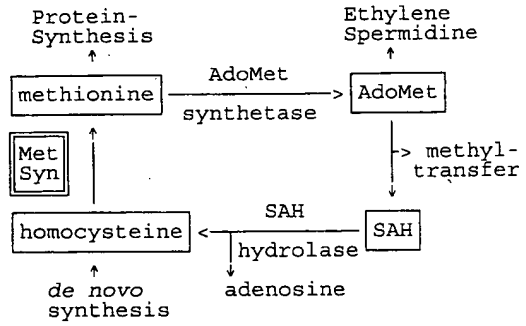

Fig. 1. Overview of the role of methionine synthase in the AdoMet-dependent cycle of biological methylation and in the *de novo* biosynthesis of methionine. Abbreviations are: MetSyn, methionine synthase; SAH, *S*-adenosyl-L-homocysteine.

tested. When *C. roseus* cell cultures are shifted from a rich culture medium containing auxin (MX medium) to aqueous 8% sucrose, the accompanying nutritional downshift and withdrawal of auxin results in transiently increased levels of messenger RNA coding for MetE, as estimated by Northern blotting, but not in significant increases in the total level of methionine synthase protein.

MATERIALS AND METHODS

Plant material. The cell suspension culture of Madagascar periwinkle (*Catharanthus roseus* L. G. Don, line CP3a), its maintenance on MX growth medium, the induction of messenger RNA synthesis by transfer into an aqueous 8% sucrose solution, and the procedure for cell fractionation have been described [15, 16]. The cell cultures for *Glycine max* (soybean), *Ammi majus* (Bishop's weed), *Petroselinum crispum* (parsley), *Dianthus caryophyllus* (carnation), and *Vitis vinifera* (grape) were from the laboratory collection or from U. Matern and J. Ebel at the University of Freiburg. The proteins from *Pinus sylvestris* (Scots pine) were extracted from 3-week-old seedlings. Methods for cell suspension culture of black Mexican sweet maize have been described previously [17]. The proteins were extracted using the protocol described in [16], and the supernatant of a 30000×*g* centrifugation for 15 min was used for the analysis. *Chlamydomonas reinhardtii* (strain CW15) was from the collection of U. Johanningmeier (University of Freiburg) and the proteins extracted in the presence of sodium dodecyl sulfate [18] were analyzed.

Antiserum and immunoblots. A cDNA fragment encoding the C-terminal 35 kDa of the *C. roseus* MetE homolog was fused in-frame with the sequence encoding the 42-kDa maltose-binding protein in vector pMal-c2 [19] to form plasmid pMal/Cros-(*metE*). The fusion protein (77 kDa) was purified by affinity chromatography on an amylose resin column and directly used for immunization of mice. The cloning and purification procedures followed the protocol provided by the manufacturer (New England Biolabs). Antiserum against the maltose-binding protein (New England Biolabs) did not react with proteins in the plant extracts. The proteins for immunoblots were separated in gels containing 0.1% sodium dodecyl sulfate and 10% polyacrylamide. After protein transfer to nitrocellulose sheets, the immunoreactions [15] were performed with a secondary antibody coupled with alkaline phosphatase (Sigma Biochemicals).

RNA isolation, cDNA libraries, and screening procedures. Poly(A)-rich RNA was isolated from *C. roseus* cell cultures induced for 7.5 h by a change from MX medium to 8% sucrose [15]. The cDNA library was constructed using 5 µg poly(A)-rich RNA and cDNA synthesis kits from Amersha (cDNA Synthesis System Plus, no. RPN1256Y) and Pharmac LKB Biotechnology (You-Prime cDNA Synthesis Kit no. 2 9273-01). After addition of *Eco*RI/*Not*I adaptors (Pharmac LKB Biotechnology), the cDNAs were ligated to *Eco*RI-c gested phage lambda NM1149 [20] and packaged with a l from Amersham (Lambda *in vitro* Packaging Kit no. N334I The phage and the screening techniques have been describ [15, 21].

DNA sequence analysis. DNA sequences were obtain with the dideoxy nucleotide chain-termination technique usi vectors, phages, and the detailed methods described in [15]. T largest cDNA was sequenced on both strands, while only part sequences were determined for the smaller cDNAs in order locate the overlap with the larger cDNA. The pTZ18R a pTZ19R vector systems and helper phage M13K07 were e ployed to obtain single-stranded DNA in *E. coli* strain JM1 (all from Pharmacia LKB Biotechnology) from the cDNAs a subcloned fragments, and the reverse sequencing primer (Boe ringer Mannheim) or custom-synthesized oligonucleotides w used for the sequence analysis. The DNA polymerization re tions were performed with (adenosine 5′-1-[^{35}S]thio)triphosph [^{35}S]dAdo*P*[S]*PP*, (37 TBq/mmol, Amersham) and modified DNA polymerase (Sequenase, United States. Biochemi Corp.). TBLASTN [22] was used for similarity searches in databases.

Protein expression in *Escherichia coli*. *E. coli* MetE: construction of plasmid pRSE562, containing the *metE* and *m* genes [23], and expression of these genes in *E. coli* str DH5αF′ have been described [6]. *C. roseus* MetE: the oligo cleotide 5′-GGGAAG CCATGG TTCTTTAGG-3′ was used introduce an *Nco*I site at the deduced start ATG (mutated ba bold and underlined). The mutagenesis was performed on sing stranded DNA obtained with helper phage M13K07 in *E.* strain RZ1032 [24] and verified by DNA sequence analysis. complete coding region plus 3′ non-translated sequences w then excised and inserted into the *Nco*I site of the express vector pQE-6 [25], retaining the optimal promotor-translati start configuration at the *Nco*I site of the vector. The plas pQE/Cros(*metE*$^+$) was maintained in *E. coli* strain M15[pRe [25] grown in Luria-Bertani medium (1% Bacto tryptone, 0. Bacto yeast extract, 0.5% NaCl, 0.2% glycerol, 0.02% Mg pH 7.5), and protein expression was induced by 2 mM isopro α-D-thiogalactopyranoside for 3 h at 28°C. Coomassie-bl stained polyacrylamide gels revealed a dominant band of expected size (85 kDa) that represented up to 40% of the t protein. It was absent in control extracts from cells contain vector pQE-6 in *E. coli* M15[pRep4].

Enzyme assays. The cell extracts were prepared by a m fication of the previously described procedure [6]. Cells of st DH5αF′ containing plasmid pRSE562 (*metE*$^+$) were grow 50 ml Luria-Bertani broth with 100 µg/ml ampicillin to an proximate total A_{420} of 3. Cells of strain M15[pRep4] contai plasmid pQE/Cros(*metE*$^+$) coding for the plant homolog w grown in the same medium, supplemented with 100 µg/ml an cillin and 25 µg/ml kanamycin, to an approximate A_{420} of 0. which point isopropyl α-D-thiogalactopyranoside was added final concentration of 2 mM, and the incubation was contin at 28°C for approximately 3 h. The cells were collected by c trifugation, washed and resuspended in 0.5 ml 50 mM Tris/ pH 7.2 and sonicated. Supernatants were collected afte 20-min centrifugation at 15000×*g* and used for enzyme acti determinations. Most assays were performed with unlabe (6*S*)-CH_3-H_4PteGlu$_3$, and the demethylation product (H_4PteG was quantified by measuring the methenyl-H_4PteGlu$_3$ formed treatment with formic acid [11], as modified by J. T. Drumm

and R. G. Matthews (unpublished work). The standard incubations (0.4 ml, duplicates) contained 50 mM Tris/HCl pH 7.2, 10 mM potassium phosphate pH 7.2, 0.1 mM $MgSO_4$, 10 mM dithiothreitol, 66 μM (6*S*)-CH_3-H_4PteGlu$_3$, prepared as described [26], and enzyme (4−47 μg and 30−40 μg protein from *E. coli* extracts containing the *C. roseus* or the *E. coli* MetE, respectively). After equilibration for 2 min at 37°C, the reactions were initiated by addition of L-homocysteine (final concentration 2 mM). They were stopped after 5 min at 37°C by mixing with 0.1 ml 5 M HCl/60% formic acid. The tubes were heated for 14 min at 84°C, allowed to return to room temperature, and centrifuged for 5 min at 16000×*g* in an Eppendorf microfuge to pellet the precipitated proteins. The absorbance at 350 nm (absorption coefficient 26500 M^{-1} cm^{-1} for methenyl-H_4PteGlu$_3$ [27]) in the supernatants was determined, and the blank was subtracted (L-homocysteine added after stop of the reaction). The extracts with *E. coli* MetE (1.8 mg protein/ml) and the *C. roseus* MetE homolog (0.9 mg protein/ml) revealed specific enzyme activities of 0.1−0.3 and 1−1.5 nkat/mg, respectively. (6*S*)-CH_3-H_4PteGlu$_3$ concentrations of 10−340 μM were used in the K_m determinations, and samples taken at 1, 2.5, 4, 5, and 10 min to ensure linearity with respect to product formation. Some assays were performed with (6*S*)-5-$^{14}CH_3$-H_4PteGlu$_3$ (0.842 mM; 22000 dpm/nmol) or (6*S*)-5-$^{14}CH_3$-H_4PteGlu$_1$ (0.325 mM; 1300 dpm/nmol) as described previously [6]; this assay quantifies the radioactive methionine formed in the reactions.

Standard molecular biology techniques. The genomic Southern blots and the Northern blots were performed as described [21].

RESULTS

MetE homolog from *C. roseus*. The MetE homolog was identified in a cDNA library from *C. roseus* by polymerase chain reaction (PCR) screening using degenerate primers that had been synthesized for another purpose. A 470-bp sequence was identified that had 45% identity with the C-terminal region of the *metE* gene from *E. coli*. Our interest in the regulation and control of one-carbon metabolism, and the complete absence of molecular information on methionine synthase enzymes in plants, led us to pursue the finding. Further screening with the fragment identified seven cDNAs in the cDNA library, ranging in size over 0.9−2.6 kbp. All contained sequences identical to the fragment and to each other, indicating that they were derived from the same mRNA. The primer sequences were present in the cDNAs, indicating that the detection of the first fragment was not an accident. The cDNAs encoded a slightly acidic protein (pI 6.05) of 765 amino acids with a calculated mass of 84857 Da. No signal sequence or N-terminal hydrophobic stretch was recognizable, suggesting that the protein was located in the cytosol. Fig. 2 shows an alignment of the deduced amino acid sequence with the partially sequenced yeast MetE homolog, originally identified as a strong promoter sequence, dP8, in yeast [28], which shows 41% identity, and with MetE from *E. coli* [6, 29, 30], with 49.5% identity. No significant similarity with *E. coli* MetH was detectable. The highly reactive Cys726 identified as candidate for the location in the active site in *E. coli* MetE [6] was conserved in the plant protein and embedded in a 12-amino-acid motif identical in the two proteins (boxed in Fig. 2).

Hybridizations with genomic DNA were performed with the complete cDNA as probe, under hybridization and washing conditions which result in strong signals at more than 70% sequence identity [31]. The small number of hybridizing bands and the small fragment sizes obtained with several restriction enzymes (Fig. 3) suggested that the *C. roseus* culture contained one, or at most a few, genes for this protein, but not a large gene family.

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a) C. roseus     MASHIVGYPRMGPKRELKFALESFWDKKSSAEDLQKV
b) E. coli     MTILN.TL.F..V.LR....K.Q..Y.AGN.TR.E.LA.
c) Yeast        MVQ.AVL.F..I..N....K.T.GY.NG.ITVDE.F..

a) AADLRSSIWKQMADAGIKYIPSNTFSYYDQVLDTATMLGAVPPRYNFAGG
b) GRE..ARH.D.QKQ...DLL.VGD.AW..H..T.SLL..N..A.HQNKD.
c) GK...TQN..LQKE..VDI....D..F......FVFVVQCHSD..TKYDL

a) EIGFDTYFSMARG---NAS-------VPAMEMTKWFDTNYHYIVPELGPE
b) SVDI..L.RIG..----R.PTGEPA---A.A.....N.....M...FVKG
c) SP-I..L.A.G..LQRK.TETEKAVD.T.L..V....S....VR.TFSKT

a) VNFSYASHKAVNEYKEAKELGVDTVPVLVGPVTFLLLSKPAKGVEKTFPL
b) QQ.KLTWTQLLD.VD..LA..HKVK...L....W.W.G.-V..EQ--.DR
c) TQ.KLNGQ.P.D.FL......IH.R...L...SY.F.G.AD.-DSLDLEP

a) LSLLDKILPVYKEVIGELKAAGASWIQFDEPTLVLDLESHQLEAFTKAYS
b) ....ND.....QQ.LA..AKR.IE.V.I...A...E.PQAW.D.YKP..D
c) ....EQL..L.T.ILSK.AS...TEV.I...V.....PANAQA.IK...T

a) ELESTLSGLNVIVETYFADIPAETYKILTALKGVTGFGFDLVRGAKTLDL
b) A.QGQ---VKLLLT...EGVTPN-LDTI...P-.Q.LHV...H.KDDVAE
c) YFGEQSNLPKITLA...GTVVPN-LDAIKG.P-.AALHV.F..APEQF.E

a) IKGGFPSGKYLFAGVVDGRNIWANDLAASLSTLQSLEGIVGKDKLVVSTS
b) LHKRL..DWL.S..LIN...V.RA..TEKYAQIK---D....RD.W.AS.
c) VVAAIGNKQT.SV.........K..FKK.SAIVNKAIEKL.A.RV..A..

a) CSLLHTAVDLVNEPKLDKEIKSWLAFAAQKVVEVNALAKALAGEKDEAFF
b) .....SPI..SV.TR..A.V...F...L..CH.LAL.RD.VNSGDTA.L-
c) S.....P...N..T...A...GFFS..T..LD..VVIT.NVS.QDVA.AL

a) SENAAAQASRKSSPRVTNQAVQKAAAALRGSDHRRATTVSARLDAQQKKL
b) A.WS.PIQA.RH.T..H.P..E.RL..ITAQ.SQ..NVYEV.AE..RARF
c) EA..KSVE..GK.KFIHDA..KARV.SIDEKMST..APFEQ..PE...VF

a) NLPVLPTTTIGSFPQTLELRRVRREYKAKKISEDDYVKAIKEEISKVVKL
b) K..AW..........T.I.TL.LDF.KGNLDANN.RTG.A.H.KQAIVE
c) ...LF..........KDI.IN.NKFNKGT..AEE.E.F.NS..E..IRF

a) QEELDIDVLVHGEPERNDMVEYFGEQLSGFAFTANGWVQSYGSRCVKPPI
b) ..R.GL.....A..........H.D..V..Q..............

a) IYGDVSRPNPMTVFWSQTAQSMTKRPMKGMLTGPVTILNWSFVRNDQPRF
b) VI..I...A.I....AKY...L.DK.V..........C...P.E.VS.E

a) ETCYQIALAIKDEVEDLEKAGINVIQIDEAALREGLPLRKAEHAFYLDWA
b) TIAK.....LR...A...A...GI.....P.........RSDWDA..Q.G

a) VHSFRITNLPLQDTTQIHTHMCYSNFNDIIHSIIDMDADVMTIENSRSSE
b) .EA...NAAVAK.D.........CE....MD..AAL....I...T...DM

a) KLLSVFREGVKYGAGIGPGVYDIHSPRIPSTEEIADRINKMLAVLDTNI
b) E..ES.-.EFD.PNE..........NV..V.W.EALLK.AAKRIPAER

a) LWVNPDCGLKTR KYAEVKPALENMVSAAKLIRTQLASAK   765
b) ........... GWP.TRA..A...Q..QNL.RG        753
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Fig. 2. Alignment of the deduced amino acid sequences for (a) methionine synthase from *C. roseus*, (b) *E. coli* MetE [6], and (c) the partial sequence of the MetE homolog from baker's yeast (*Saccharomyces cerevisiae*) [28]. The symbols used are: dashes, gaps for optimal alignment; dots, sequence identity; underlined, conservative exchanges. Shown in the box is a conserved 12-amino-acid motif containing the putative active site Cys726 of *E. coli* MetE [6].

Heterologous expression and comparison of enzyme properties with methionine synthase enzymes from *E. coli*. The plant protein was expressed in *E. coli* to test the function predicted from the similarity to *E. coli* MetE, and to investigate the enzyme properties. Extracts containing the plant protein had methionine synthase activity with CH_3-H_4PteGlu$_3$ as methyl donor, but not with CH_3-H_4PteGlu$_1$, corresponding to the properties of *E. coli* MetE and the yeast homolog, but not to *E. coli* MetH, which accepts both methyl donors. Control *E. coli* extracts from strain M15[pRep4], which has a chromosomal *metE* gene but contains the vector plasmid pQE6 without the cloned plant DNA, had no significant activity because the bacterial MetE is repressed during growth in rich media containing methionine. MetH activity requires AdoMet and a reducing system that may be either cobalamin/thiol [8] or two flavoproteins plus NADPH [9]. The addition of AdoMet (19 μM) and aquacobalamin (50 μM) to the assay with the plant protein had no effect on the activity with CH_3-H_4PteGlu$_3$, and also did not lead to activity

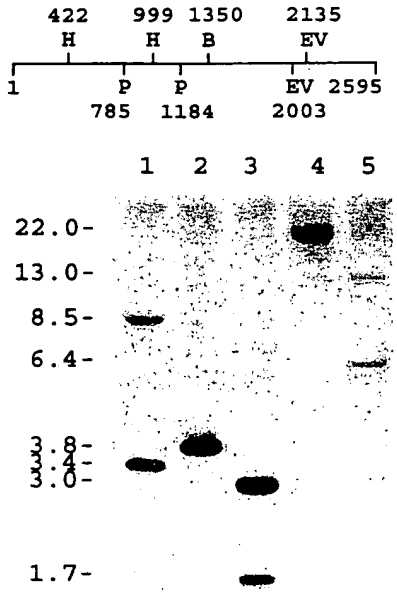

Fig. 3. Genomic Southern blot with *C. roseus* metE cDNA. Top: diagram of the 2.6-kbp cDNA with the positions of the restriction sites (distance in bp from the 5′ end of the cDNA) for the enzymes used in the digestion of the genomic DNA. B, *Bam*HI; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I. Bottom: genomic Southern blot using the complete cDNA as the probe. The restriction enzyme used to cleave the genomic DNA in each lane was: 1, *Eco*RI; 2, *Eco*RV; 3, *Hin*dIII; 4, *Bam*HI; 5, *Pst*I. The weakness of the bands in lane 5 reflects a poor digestion of the plant DNA with *Pst*I.

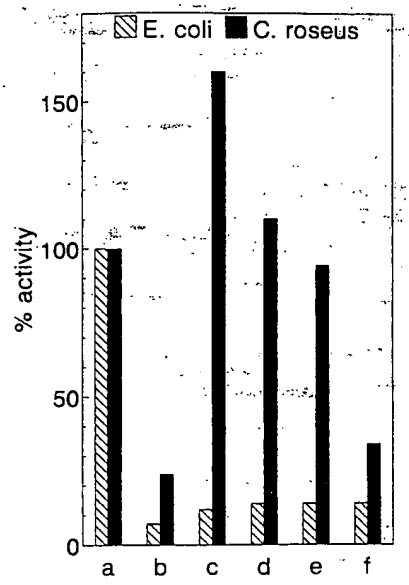

Fig. 4. Comparison of the properties of the *E. coli* MetE and *C. seus* methionine synthase enzyme activities. (a) Assay of the enzy under the standard assay conditions described in Materials and Meth (b) dithiothreitol omitted; (c) phosphate buffer omitted; (d) Mg omitted; (e) $MnSO_4$ added instead of $MgSO_4$; (f) 3 mM EDTA ad divalent cations omitted. The experiments were performed with (CH_3-H_4PteGlu$_3$ as methyl donor. The activities in the complete as were 1.18 nkat/mg for the plant enzyme and 0.23 nkat/mg for the *E.* enzyme. The lower activity of the *E. coli* MetE reflects the repres of this enzyme during growth in rich media containing methionine cause the native promoter is present in pRSE562.

with CH_3-H_4PteGlu$_1$. The results indicated that the cloned protein was a cobalamin-independent methionine synthase of the MetE type.

Like the bacterial protein, the plant enzyme was sensitive to the omission of thiols in the assay mixture (compare Fig. 4 a and b). *E. coli* MetE contains seven thiols, at least one of which is highly reactive [6], and thiols may be required to reduce disulfide bonds and prevent inactivation during the enzyme assay. The further analysis revealed some interesting differences between the plant enzyme and *E. coli* MetE (characterized in [7]). The bacterial enzyme requires phosphate in millimolar concentrations, and omission of phosphate lead to a strong reduction of the activity (Fig. 4c). However, omission of phosphate buffer stimulated the plant MetE activity, suggesting that the phosphate concentration in the standard assay for *E. coli* MetE (10 mM) was inhibitory to the plant enzyme. The bacterial enzyme needs Mg^{2+} for full activity, and replacing Mg^{2+} by Mn^{2+} leads to a decreased activity, but no such effect was observed with the plant protein (Fig. 4 e). The plant enzyme in fact required no addition of divalent cations for full activity (Fig. 4 d), and an excess of EDTA was necessary to reduce the activity to about 35% of the control levels (Fig. 4 f). The results indicated that the plant and the *E. coli* enzymes differed in their requirements for cations, although experiments with a highly purified plant protein would be necessary to determine whether these differences are seen with purified enzymes.

The K_m of the plant enzyme for CH_3-H_4PteGlu$_3$ was 28 μM, a value higher than the 4.7 μM figure obtained for *E. coli* MetE [7], but considerably lower than the 0.4 mM value reported for the yeast MetE homolog [1]. K_m values from other plant enzymes [11] were not available because kinetic experiments were performed only with CH_3-H_4PteGlu$_1$, a methyl donor that was not accepted by the cloned *C. roseus* protein. The K_m for L-homocysteine could only be estimated because the insensitivity

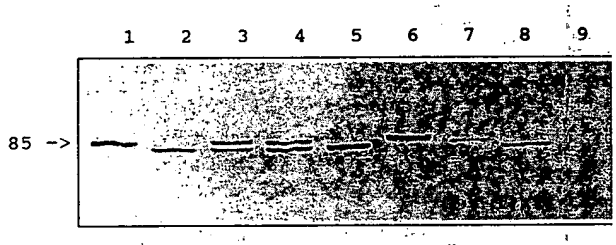

Fig. 5. Immunoblot for Met-E type methionine synthases in *C. ro* and in other plants. 1, *Catharanthus roseus* (Madagascar periwin 2, *Glycine max* (soybean); 3, *Ammi majus* (Bishop's weed); 4, *Petro num crispum* (parsley); 5, *Dianthus caryophyllus* (carnation); 6, *vinifera* (grape); 7, *Pinus sylvestris* (Scots pine); 8, *Zea mays* (mai 9, *Chlamydomonas reinhardtii*. Each slot contained 50 μg protein f a 30000×*g* supernatant of the corresponding extract. The size of th *roseus* protein, indicated on the left margin, is 85 kDa.

of the available assay did not permit reliable determination very low substrate concentrations, but the data indicated a value of less than 10 μM. The K_m for homocysteine of the y enzyme was reported to be higher (22 μM) [1].

The cloned plant methionine synthase is a soluble pro and ubiquitous in plants. Immunoblots with *C. roseus* extr revealed a single protein band of 85 kDa (Fig. 5, lane 1), a corresponding to the molecular mass deduced from the quence; the same size was obtained with the protein expres from the deduced start ATG in *E. coli* (Fig. 6A, lane b, at right). It was detected only in the soluble protein frac (100000×*g* supernatant), and no immunoreactive proteins w present in the fractions containing membranes or organelles. results indicated that the protein was cytosolic, in agreem with the lack of a signal peptide or an N-terminal hydroph

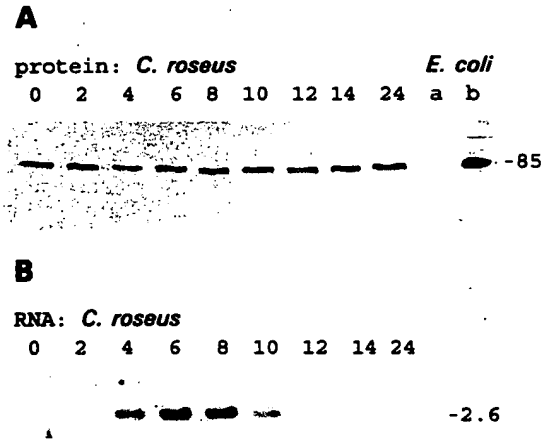

Fig. 6. Patterns of expression of MetE protein (A) and *metE* transcript (B) in *C. roseus* cell cultures after transfer of a 7-day-old culture from MX medium to a solution of aqueous 8% sucrose. The numbers indicate hours after the medium change. (A) Immunoblots with the antiserum to the *C. roseus* MetE homolog. Each slot contained 50 μg plant protein extract from a 30000×*g* supernatant of cells collected from the culture at the indicated time. The lanes a and b on the right of the figure show immunoblots of 10 μg protein from *E. coli* cell extracts: (a) control strain M15[pRep4] containing vector pQE-6; (b) strain M15[pRep4] containing plasmid pQE/Cros(*metE*$^+$). The position of the 85-kDa MetE homolog is indicated on the right. (B) Northern blots (10 μg total RNA/slot). The position corresponding to the 2.6-kb transcript is indicated at the right.

stretch in the protein sequence. Experiments with other plants, including gymnosperms and monocots, showed that all contained cross-reacting proteins of comparable size. The proteins in soybean and carnation were slightly smaller than in *C. roseus* (Fig. 5, lane 2 and 5). Doublets representing both size forms were detected in Bishop's weed and parsley (lanes 3 and 4). No immunoreactive protein was detected in *Chlamydomonas reinhardtii* (lane 9) in these experiments.

Stress-induced transient increase of transcription. The regulation of MetE expression was investigated with *C. roseus* cell cultures under stress conditions caused by transfer of the cells from the standard MX growth medium to a solution of aqueous 8% sucrose (Fig. 6). Such a transfer leads to deprivation of nitrogenous compounds in the medium, an increased concentration of the sucrose carbon source, and deprivation of the auxin growth hormone present in MX medium (2,4-dichlorophenoxyacetic acid). After the shift, the transcript analysis (Fig. 6B) revealed the induction of an RNA corresponding to the size of the largest cDNA in the library (2.6 kbp), indicating that the cDNA was either full-length or very close to it. Fig. 6B also shows that the amount of the 2.6-kb RNA was very low in non-induced cells, and that it was induced strongly 4–8 h after the change of the medium, and declined thereafter. The immunoblots for the protein, however, revealed no significant change in the amount of immunodecorated protein during the induction period (Fig. 6A). The significance of this finding is not clear at present, but one possible explanation could be that the regulation of protein expression is under independent control. The same phenomenon was observed with the two other enzymes involved in the regeneration of active methyl groups (AdoMet synthetase and *S*-adenosyl-L-homocysteine hydrolase; J. E., unpublished data).

DISCUSSION

To the best of our knowledge, this is the first report of the molecular characterization of a plant methionine synthase and of its functional identification by heterologous expression. The *C. roseus* enzyme shared sequence similarities and properties with *E. coli* MetE [e.g. cobalamin independence and a requirement for polyglutamate forms of (6*S*)-CH_3-H_4folate], but there were also interesting differences (e.g. the lack of a requirement for added phosphate and magnesium ions for the activity of the plant enzyme). The yeast MetE homolog was also Mg^{2+}-independent and inactive with (6*S*)-CH_3-H_4PteGlu$_1$, but required phosphate like *E. coli* MetE [1]. The significance with respect to function or regulation of the enzyme activity *in vivo* remains to be investigated.

Plants synthesize L-homocysteine *de novo*, and therefore methionine synthases are used for both methionine biosynthesis and regeneration of the methyl group, with estimates that ≥75% of the amino acid synthesized is used to provide methyl groups for the multitude of methylation reactions in plants [32, 33]. It is difficult to estimate at present whether the cloned protein serves in both pathways or whether different isozymes are used for biosynthesis and for remethylation of homocysteine. In contrast to the protein cloned from *C. roseus*, partially purified preparations from other plants were reported to have significant activity with CH_3-H_4PteGlu$_1$ (15% of the activity with CH_3-H_4PteGlu$_3$) [34]. Although it cannot be rigorously excluded at present that the substrate requirements in the plant preparations were influenced by factors absent in the *E. coli* extracts, the difference suggests that other methyltetrahydrofolate-dependent methionine synthases may be present in some plants. The presence of two (or more) enzymes could also explain the reported dependence on Mg^{2+} and phosphate observed with CH_3-H_4PteGlu$_1$ as substrate (reviewed in [11]); future experiments will adress the question whether methionine synthase activities with these properties are present in *C. roseus*. The presence of protein doublets in some plants (Fig. 5) suggests isozymes and these may differ in their properties. These possibilities can be resolved only after separation and complete purification of any isozymes.

The complexity of methionine formation in plants is also evident from the presence of three other enzymes that synthesize methionine from L-homocysteine. However, these enzymes utilize other methyl donors (AdoMet, *S*-methylmethionine, or methylthioadenosine) that are derived from methionine in the first place, and therefore these reactions do not represent net biosynthesis of the amino acid [11]. The protein described in the present work is different, and the availability of cloned sequences provides a tool to unravel the complexities of *de novo* synthesis of methionine in plants.

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